

# Mitochondrial Phylogeography of Vegetable Pest *Liriomyza sativae* (Diptera: Agromyzidae): Divergent Clades and Invasive Populations

SONJA J. SCHEFFER AND MATTHEW L. LEWIS

Systematic Entomology Laboratory, USDA-ARS-PSI, Building 005, Room 137, BARC-W, 10300 Baltimore Avenue, Beltsville, MD 20705

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**ABSTRACT** The leafmining fly *Liriomyza sativae* Blanchard is an important polyphagous pest of vegetable crops in the United States and around the world. Phylogeographic analysis of mitochondrial cytochrome oxidase I sequence variation indicates that *L. sativae* harbors distinct mitochondrial clades suggestive of the presence of cryptic species. Two of the major mitochondrial clades exhibited polyphagy, with members feeding on hosts in at least three plant families. Only one of the major clades was present in introduced populations, causing total mitochondrial variation exhibited by introduced populations to be considerably reduced compared with that observed within the native range.

**KEY WORDS** phylogeography, cryptic species, leafminer, invasive species, agromyzid

PHYLOGEOGRAPHIC ANALYSIS within species or among closely related species provides a powerful method for investigating evolutionary and biogeographic history (Avice 2000). Phylogeographic analysis can be used to delineate morphologically cryptic species and to identify possible causes of divergence (Avice 2000). In invasive species, phylogeographic analysis may be used to identify the geographic origins of invasive populations and to illuminate the mode or pathway of geographic spread (Slade and Moritz 1998, Scheffer and Grissell 2003). In agricultural pests, information on cryptic species and geographic origins of invasive populations may be critical to understanding pest populations, designing effective management strategies, and preventing further spread of the pest (Rosen 1978, Schauf and LaSalle 1998).

*Liriomyza sativae* Blanchard is a leafmining fly that is a polyphagous pest of many vegetable and flower crops (Spencer 1973, 1990; Parrella 1982). It has been recorded from nine plant families, although its preferred hosts tend to be in the Cucurbitaceae, Fabaceae, and Solanaceae (Spencer 1973, 1990). There is a long history of taxonomic confusion regarding *L. sativae*, making the literature on this species before the 1970s difficult to interpret. *L. sativae* has been inadvertently redescribed at least seven times from various hosts and locations (Spencer 1973, Parrella 1982, Spencer and Steyskal 1986). Additionally, the morphological similarity of *L. sativae* to other pest species has caused numerous misidentifications to obscure our understanding of various aspects of the biology and ecology of this species (Spencer 1973, Parrella 1982, Parrella and Keil 1984).

The endemic range of *L. sativae* includes regions of both North and South America; *L. sativae* was origi-

nally described in Argentina in 1938 and was present in several vegetable growing areas in the United States at about the same time (Spencer 1973, Parrella 1982). Recent molecular data have shown that widespread endemic distributions of this sort may contain highly divergent cryptic lineages (Omland et al. 2000, Scheffer 2000, Scheffer and Lewis 2001), possibly representing cryptic species (Avice 2000). With respect to polyphagous pest *Liriomyza* species, there are anecdotal reports of populations from different locations differing in preferred host plants. Scientists have even been warned that "flies identified as the same species . . . may not necessarily exhibit similar characteristics, even though they may be from the same host" (Parrella 1982). Recent investigations of the sister species of *L. sativae*, the polyphagous leafminer *Liriomyza trifolii* (Burgess), indicate that this species contains a distinct and reproductively isolated host race on peppers (Morgan et al. 2000, Reitz and Trumble 2002, unpublished data).

Although *L. sativae* is of New World origin, it has spread worldwide via international trade in vegetables and flowers (Parrella 1987, Rauf et al. 2000, Andersen et al. 2002, Chen et al. 2003). Introduced populations are prone to outbreaks and are often difficult to control (Parrella 1987, Chen et al. 2003). An understanding of the makeup and origin of invasive populations would aid management efforts and provide general information on invasion biology.

The purpose of this study was to investigate the global phylogeographic structure within *L. sativae*. Specifically, we address four questions: 1) Are highly diverged mitochondrial clades present within *L. sativae*? 2) What are the host affiliations of such clades? 3) What are the geographic distributions of such

Table 1. Collection information for *L. sativae* specimens

Location	Date	n	Stage	Host	Collector(s)
New World					
Colombia					
Valle del Cauca	Nov. 2000	5	Larva	Kidney bean	J. Gueveno
Guatemala					
El Jocotillo	Dec. 2001	3	Adult	Unknown	C. MacVean, C. Cardona
Amatitlan	April 2001	3	Adult	Bean	C. MacVean, K. Tay
Chiquimula	April 2001	3	Adult	Tomato	C. MacVean, G. Acosta
Baha Verapaz	May 2001	2	Adult	Tomato	C. MacVean, C. Cardona
Baha Verapaz	May 2001	2	Adult	Cucumber	C. MacVean, C. Cardona
Honduras					
Mirador	March 2001	4	Larva	Tomato	R. Cave
United States					
Arizona, Yuma Exp. Sta.	2000	5	Adult	Melon (Trapped)	J. Palumbo
California, Davis	1999	5	Adult	Bean	L. Godfrey, C. Black
Colorado, Bailey	June 1999	7	Adult	Lupine (swept)	S. Scheffer
Florida, Bradenton	April 2001	2	Adult	Weeds (swept)	S. Scheffer
Florida, Homestead	April 2001	3	Adult	Tomato (swept)	S. Scheffer
Florida, Disney Epcot Center	Sept. 1995	2	Adult	Colony	D. Wietlisbach, F. Pettit
Florida, Disney Epcot Center	Aug. 2000	2	Adult	Colony	D. Wietlisbach, F. Pettit
Venezuela, Monagas	March 2001	3	Larva	Bean	J. Gueveno
Old World					
China					
Beijing	Aug. 2001	4	Adult	Bean	X.-L. Chen
Beijing	Aug. 2001	4	Adult	Chrysanthemum	X.-L. Chen
Shandong Prov., Tengzhou	Sept. 1998	4	Adult	Bean	L. Kang, J. LaSalle
Egypt					
Saads Region	Feb 1999	7	Adult	Fava bean	S. El-Serwy
Al-Aiat, Giza	March 1999	1	Adult	Bean	S. El-Serwy
Sids, Beni Sueif	Jan. 1999	3	Adult	Lentil bean	S. El-Serwy
Israel					
Gilat Exp. Sta.	May 2000	2	Adult	(Vaccumed)	P. Weintraub
Malaysia					
Malacca	Oct. 1998	4	Adult	Bean	J. LaSalle
Selanger	July 1998	3	Adult	Gourd	J. LaSalle
Philippines					
Ilocos Sur	Nov. 2000	4	Adult	Tomato	R. Joshi, N. Baucas
Mountain Prov.	Sept. 2000	2	Adult	Bean	R. Joshi, N. Baucas, G. Sacla
Saudi Arabia	2002	5	Adult	Unknown	A. Al Jabr
Sri Lanka					
Etulgama	Sept. 1998	4	Adult	Bean	A. Wijesekara
Gira Kotte	May 1998	4	Adult	Honey melon	A. Wijesekara
Gannoruwa	Aug. 1998	1	Adult	Zucchini	A. Wijesekara
Magastota	March 1998	1	Adult	Zucchini	A. Wijesekara
Vietnam					
An Hai District	July 1998	6	Adult	Long bean	J. LaSalle

clades? and 4) What are the structures and sources of invasive populations?

### Materials and Methods

*L. sativae* specimens were obtained from a variety of locations and hosts around the world (Table 1). Larval, pupal, and adult specimens were preserved for study in 95% ethanol and stored at  $-80^{\circ}\text{C}$ . Before DNA extraction, morphological features of specimens were checked by S.J.S. to ensure that only *L. sativae* were included in the study. Generally, adult specimens were used, but some collections contained only larvae. Although larval *Lirionomyza* specimens cannot be determined to species from morphological characters, such specimens of *L. sativae* and *L. trifolii* can be distinguished from those of most other pest species by the shape of the posterior spiracles. Because larval stages of *L. sativae* and *L. trifolii* cannot be distinguished from each other morphologically, we used mitochondrial sequence data to determine species

identity when larval specimens were used (Table 1). Voucher specimens of adults from several of the populations have been deposited in the National Museum of Natural History in Washington, DC.

Extraction of total nucleic acids from single specimens was accomplished using the DNeasy insect protocol B (QIAGEN, Valencia, CA). Polymerase chain reaction (PCR) amplification of most of cytochrome oxidase I (COI) was carried out using a Mastercycler Gradient thermocycler (Eppendorf Scientific, Inc., Westbury, NY) with a touchdown amplification program: initial denaturation at  $92^{\circ}\text{C}$  for 2 min, followed by two touchdown cycles from  $58$  to  $4^{\circ}\text{C}$  ( $10$  s at  $92^{\circ}\text{C}$ ,  $10$  s at  $58-46^{\circ}\text{C}$ ,  $2$  min at  $72^{\circ}\text{C}$ ),  $29$  cycles of  $10$  s at  $92^{\circ}\text{C}$ ,  $10$  s at  $45^{\circ}\text{C}$ ,  $2$  min at  $72^{\circ}\text{C}$ , and a final extension step for  $10$  min at  $72^{\circ}\text{C}$ . A single fragment of  $1533$  bp resulted from PCR amplification with primers C1-J-1535 (5'-ATTGGAACCTTTATATTTTATTTTGG-3') and TL-N-3017 (5'-CTTAAATCCATTGCACTA-ATCTGCCATA-3') (primer names follow the system of Simon et al. 1994). PCR product was purified using

the QIAquick PCR purification kits (QIAGEN). Because of the large number of specimens in the study, we chose to sequence only a portion of the amplified fragment, 550 bp of the 3' region of COI, by using the internal primer C1-J-2441 (5'-CCTACAGGAATTA-AAATTTTTCAG TTGATTAGC-3') and the external primer TL-N-3017 (see above). Most sequences were confirmed by sequencing in both directions. Occasionally, if one of the primers failed to sequence well, we used unconfirmed sequence data, but only when the electropherograms were very clean. All unconfirmed sequence data were corroborated by identical sequences from other individuals in the study. Sequences were deposited in GenBank under accession numbers AY697731–AY697843.

ABI Big Dye Terminator sequencing kits (Applied Biosystems, Foster City, CA) were used for all sequencing reactions with the modification that volume of all reaction components was reduced to 25% of that recommended by the manufacturer. Sequence data were obtained by analyzing samples on an ABI 377 Automated DNA sequencer. Contig assembly, as well as the final alignment of consensus sequences, was accomplished using the program Sequencher (Gene Codes Corp., Ann Arbor, MI). Identical sequences were removed from the final data set so that each haplotype was only represented once.

Maximum parsimony analysis was performed using the heuristic search feature of PAUP\* 4.0b8 (Swofford 2001) with 100 random addition replicates. Bootstrapping was performed with 1000 pseudoreplications of the data set. Uncorrected pairwise distances presented as ranges were calculated using PAUP\*. Outgroups for analysis of this dataset included *L. trifolii*, the sister species to *L. sativae* (Spencer 1990; S.J.S., unpublished), and a slightly more distant congener *Liriomyza huidobrensis* (Blanchard).

## Results

Within *L. sativae*, 24 haplotypes were found from 110 individuals from eight hosts and 13 countries (Table 1). Maximum parsimony analysis of this data set resulted in 538 equally parsimonious trees, one of which is presented in Fig. 1. The strict consensus of the equally parsimonious trees indicates that variation among these 538 trees is entirely due to variation in arrangements within major clades; branches recovered in the strict consensus are indicated in bold in Fig. 1. Three major clades were found within *L. sativae*: *sativae*-A, *sativae*-L, and *sativae*-W (Fig. 1). Uncorrected pairwise distances among distinct haplotypes within these major clades range from 0.2 to 1.3%, whereas distances between clades range from 2.5 to 8.8% (Table 2). Distances from the ingroup to the outgroups range from 8.3 to 12.8%.

Host affiliation of the three major clades indicates little structuring of the mitochondrial variation by host plant. Clades *sativae*-A and *sativae*-W were each collected from bean (Fabaceae), tomato (Solanaceae), and members of the Cucurbitaceae. However, mem-

bers of clade *sativae*-L were exclusively swept from lupine (Fabaceae).

The mitochondrial variation present within *L. sativae* exhibits some geographic structure: *sativae*-A was found only in Florida, Guatemala, and Honduras; *sativae*-L was found only in Colorado on wild lupines; and *sativae*-W was widespread, being found in Florida, California, Arizona, and all invasive Old World populations sampled in this study (Fig. 1). Eleven of the 15 haplotypes found in clade *sativae*-W were present in introduced Old World samples (haplotypes in rectangles, Fig. 1; Table 3), and five of these were unique to Old World samples (Table 3). Mitochondrial variation across sampled New World populations as measured by uncorrected pairwise distances ranged to 8.8%, whereas variation across sampled Old World populations ranged only to 1.2%.

## Discussion

Within *L. sativae*, several distinct mitochondrial clades were observed with pairwise distances between them ranging from 2.5 to 8.6% (Table 2). This distance is similar in magnitude to those observed between closely related agromyzid species (Scheffer and Wiegmann 2000) and suggests that *L. sativae* may harbor cryptic species. This possibility is not surprising given what we know about the endemic range, which seems to have been highly disjunct, with both North and South American populations (Spencer 1973). The deep mitochondrial divergence seen within *L. sativae* is very similar to that observed in what once was considered *L. huidobrensis*. In that species, what seemed to be a single polyphagous pest present in both North and South America was found to have a deep mitochondrial divergence of 4.6–5.3% (Scheffer 2000). The presence of two highly diverged lineages was corroborated by fixed differences in two nuclear genes, Elongation Factor 1- $\alpha$  (EF-1 $\alpha$ ) and a Beta-tubulin intron (b-tub), and the taxonomy was changed to reflect the presence of two distinct species, *L. huidobrensis* in South America and *L. langei* Frick in California and Hawaii (Scheffer and Lewis 2001). Preliminary sequence data from EF-1 $\alpha$  and b-tub in *L. sativae* are inconclusive regarding the structure of genetic variation within this species (S.J.S., unpublished).

Whether the diverged mitochondrial clades within *L. sativae* represent distinct cryptic species cannot be determined with evidence only from a single mitochondrial gene. It is possible that the observed divergences represent polymorphisms within populations that freely interbreed in sympatry (Avice 2000). Such highly diverged mitochondrial polymorphisms could be caused by recent mixture of reproductively compatible but previously disjunct populations. Additionally, because the sampling in this study is almost exclusively from pest populations in only a few regions of the endemic range, what seem to be deep splits may instead be artifacts of limited sampling. For example, the *sativae*-L population was swept from wild lupines in a small field in the Rocky Mountains of Colorado;

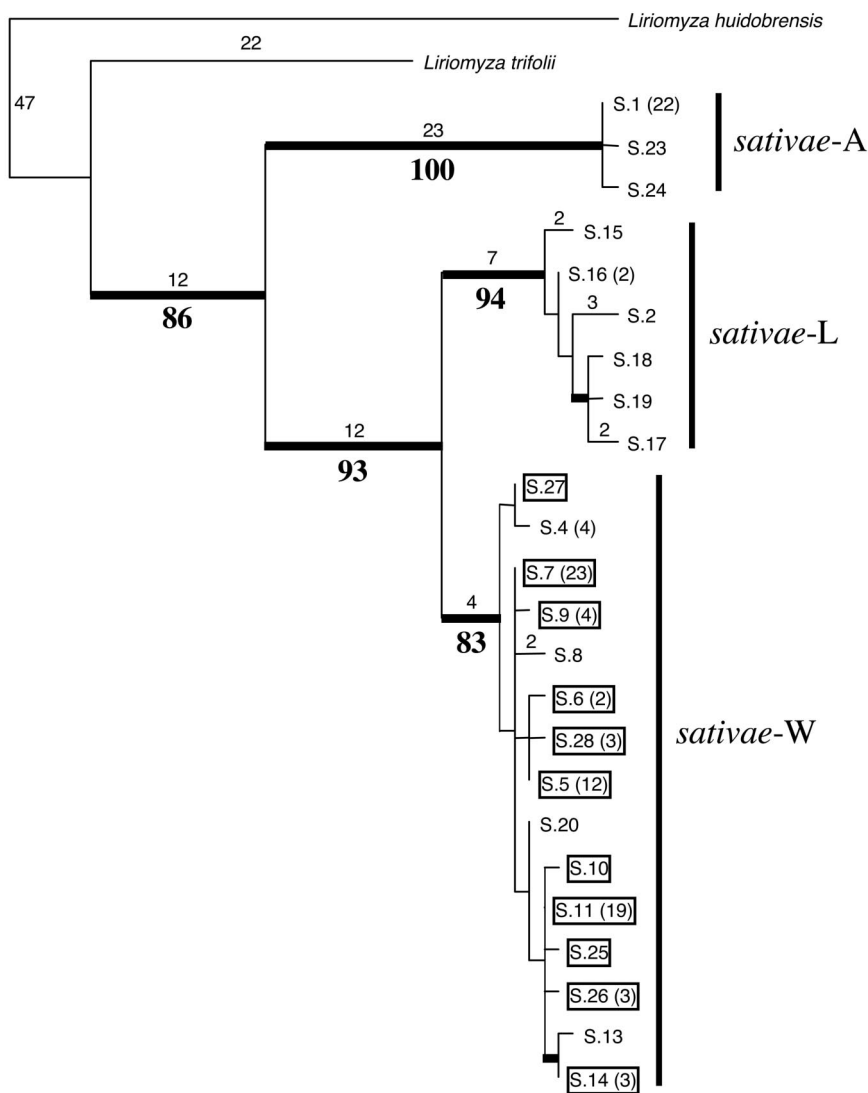


Fig. 1. One of 538 equally parsimonious phylograms of relationships among haplotypes of *L. sativae*. Estimated branch lengths ( $>1$ ) are shown above branches, whereas bootstrap values are shown below. Branches in bold indicate those recovered in the strict consensus of the 538 equally parsimonious trees. The number of individuals carrying a haplotype ( $>1$ ) is given in parentheses. Haplotypes present in geographic regions outside of the Americas are enclosed in a rectangular box. Collection locations and host affiliations of haplotypes are listed in Table 3.

that this population is comprised of unique haplotypes not found in other populations suggests that more diversity may be present within the Americas than was uncovered by this study. It is possible that samples

from other regions between Florida and California would contain variants falling between *sativae*-L and *sativae*-W, reducing the apparent distinctiveness of these lineages.

It is unlikely that uneven sampling can explain all of the observed deep divergences seen within *L. sativae*. For example, the divergence of 7.6–8.8% between *sativae*-A and *sativae*-W+L is very large and seems to be confirmed by a biological difference; PCR amplification and sequencing indicate that all members of the *sativae*-A clade are infected with a single *Wolbachia* subgroup A strain, whereas none of the members of the *sativae*-W+L clade have this infection (M.L.L. and S.J.S., unpublished). *Wolbachia* infections in in-

Table 2. Ranges of percentage of uncorrected pairwise distances among distinct haplotypes based on 529 bp of COI for major clades within *L. sativae*

Clade	<i>sativae</i> -A	<i>sativae</i> -L	<i>sativae</i> -W
<i>sativae</i> -A	0.2–0.4		
<i>sativae</i> -L	7.6–8.4	0.4–1.3	
<i>sativae</i> -W	7.6–8.8	2.5–3.8	0.4–1.1

Interclade distances indicated in bold.

Table 3. Collection locations and host plants for mitochondrial clades and haplotypes found within *L. sativae* (N = 110)

Clade	Haplotype	N	Location (host, no. of specimens)
<i>sativae</i> -A	S-1	22	FL (Epcot colony, 4; swept, 3); Guatemala (bean, 2; tomato, 5; cucumber, 2; unrecorded, 2); Honduras (tomato, 4)
<i>sativae</i> -A	S-23	1	Guatemala (unrecorded, 1)
<i>sativae</i> -A	S-24	1	Guatemala (tomato, 1)
<i>sativae</i> -L	S-2	1	CO (swept lupine, 1)
<i>sativae</i> -L	S-15	1	CO (swept lupine, 1)
<i>sativae</i> -L	S-16	2	CO (swept lupine, 2)
<i>sativae</i> -L	S-17	1	CO (swept lupine, 1)
<i>sativae</i> -L	S-18	1	CO (swept lupine, 1)
<i>sativae</i> -L	S-19	1	CO (swept lupine, 1)
<i>sativae</i> -W	S-4	4	AZ (melon, 1); Colombia (bean, 3)
<i>sativae</i> -W	S-5	12	AZ (melon, 1); Colombia (bean, 2); Egypt (bean, 2); Israel (vacuumed, 1); Sri Lanka (melon, 3); Venezuela (bean, 3)
<i>sativae</i> -W	S-6	2	AZ (melon, 1); Sri Lanka (bean, 1)
<i>sativae</i> -W	S-7	23	CA (bean, 1); China (bean, 5; chrysanthemum, 3); Egypt (bean, 2); Malaysia (bean, 1; gourd, 2); Philippines (bean, 1; tomato, 1); Saudi Arabia (unknown, 1); Sri Lanka (bean, 1; melon, 1; zucchini, 1); Vietnam (bean, 3)
<i>sativae</i> -W	S-8	1	FL (swept, 1)
<i>sativae</i> -W	S-9	4	AZ (melon, 1); FL (swept, 1); Philippines (tomato, 1); Saudi Arabia (unknown, 1)
<i>sativae</i> -W	S-10	1	Sri Lanka (bean, 1)
<i>sativae</i> -W	S-11	19	CA (bean, 3); China (bean, 3; chrysanthemum, 1); Egypt (bean, 6); Israel (vacuumed, 1); Philippines (tomato, 1); Sri Lanka (bean, 1; zucchini, 1); Vietnam (bean, 2)
<i>sativae</i> -W	S-13	1	CA (bean, 1)
<i>sativae</i> -W	S-14	3	Egypt (bean, 1); Saudi Arabia (unknown, 2)
<i>sativae</i> -W	S-20	1	AZ (melon, 1)
<i>sativae</i> -W	S-25	1	Saudi Arabia (unknown, 1)
<i>sativae</i> -W	S-26	3	Malaysia (bean, 3)
<i>sativae</i> -W	S-27	1	Malaysia (gourd, 1)
<i>sativae</i> -W	S-28	3	Philippines (bean, 1; tomato, 1); Vietnam (bean, 1)

sects are known to result in reproductive incompatibilities between infected and uninfected populations (Stouthamer et al. 1999, Stevens et al. 2001). The effects of infection in *L. sativae* populations are not yet known. The two highly diverged mitochondrial clades *sativae*-A and *sativae*-W are present in sympatry in Florida; future work will be able to investigate whether gene flow between these clades is occurring.

The host affiliations of the clades found in the current study generally confirm the polyphagous nature of *L. sativae* populations. Clades *sativae*-A and *sativae*-W were both reared from Fabaceae, Solanaceae, and Cucurbitaceae, the three host families previously reported to be most preferred by *L. sativae* (Spencer 1973), although a single collection of clade *sativae*-W came from chrysanthemum (Asteraceae) in China. The flies swept from lupine (Fabaceae) in Colorado seem to represent a host-associated group, but because geography and host are confounded (no other *L. sativae* were collected from this location) clade *sativae*-L may represent geographic structuring that happened to be associated with lupine. Collecting from additional hosts and locations within the native range of *L. sativae* will further explore the associations of geography and host affiliation with phylogeographic structure in this species.

*L. sativae* is one of several pest leafminers that has been inadvertently introduced to locations outside of the Americas as the result of global trade (Spencer 1973; Parrella 1982, 1987; Minkenberg 1988; Rauf et al. 2000; Andersen et al. 2002). Introduced populations of

*L. sativae* sampled by the current study exhibit only a fraction of the mitochondrial variation present in the Americas, a result similar to that found in other pest *Liriomyza* species (Scheffer 2000, 2005). All introduced *L. sativae* sampled to date belong to the clade *sativae*-W (Fig. 1), a clade that is also widespread within North America, having been recovered from major vegetable growing regions in California, Florida, and Arizona (Table 2). The clade *sativae*-A, a pest in Florida, Guatemala, and Honduras, has not yet been found in other regions. With the present data, it is not possible to determine the geographic origin(s) of either clade *sativae*-A or clade *sativae*-W, but increased sampling of nonpest populations within the Americas can be expected to prove informative in this regard.

This investigation establishes that distinct mitochondrial clades suggestive of cryptic species are present within the currently recognized *L. sativae*. Only one clade seems to be invasive on a worldwide scale, indicating that even among pest populations, lineages can vary in propensity to invade new regions. Additional study of molecular variation and behavior of this leafmining fly will provide more information on species status, pest biology, invasion processes, and speciation.

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